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PRINCIPAL INVESTIGATOR: Andrea Baillo

CONTRACTING ORGANIZATION: Wayne State University
Detroit, MI 48201

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Andrea Baillo

E-Mail: bailloa@karmanos.org

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14. ABSTRACT
Inflammatory breast cancer (IBC) is one of the most lethal forms of breast cancer. A strong correlation has been found between inflammatory breast cancer and high levels of the growth factor amphiregulin. Amphiregulin is known to activate the epidermal growth factor receptor (EGFR) which turns on genes involved in a number of functions including cell growth. Self-sustained activation of EGFR by amphiregulin is called an amphiregulin autocrine loop. An amphiregulin autocrine loop has been suggested to contribute to tumor growth and metastasis. Therefore, we propose to determine the potential role of an amphiregulin autocrine loop in the progression of inflammatory breast cancer. To perform our experiments, we will utilize the SUM149 cell line which was derived from an aggressive inflammatory breast cancer. These cells have an amphiregulin autocrine loop which makes them a good model for our studies. Amphiregulin and other closely related growth factors are synthesized as large membrane associated forms which can be shed from the membrane and released into the microenvironment. Some studies have suggested that these growth factors require shedding in order to interact with the EGFR. This is the reason clinical drugs have been made to prevent the shedding of membrane bound growth factors. However, there is also evidence suggesting that some membrane precursors are biologically active. We found that SUM149 cells have high levels of membrane bound amphiregulin and our data suggest that the membrane bound form may be able to activate the EGFR which could have important implications on cancer progression. Therefore our first aim will be to determine whether membrane bound amphiregulin is biologically active and whether it is required for an amphiregulin autocrine loop. We will test this aim by isolating the membrane bound form of amphiregulin from SUM149 cells and determine how it affects the growth of normal human mammary epithelial cells. We will also inhibit release of amphiregulin from the membrane in order to determine how this affects EGFR activity and cell growth. Growth factors related to amphiregulin are known to promote varying degrees of EGFR recycling or degradation. If EGFR is recycled back to the membrane, it can become activated again. Therefore, degradation of EGFR is important to maintain normal cellular EGFR levels and to prevent abnormal cell growth. The effects of amphiregulin on EGFR degradation have not yet been examined. Therefore, our second aim is to determine if amphiregulin is contributing to the high levels of EGFR observed in inflammatory breast cancer by decreasing EGFR degradation or increasing recycling. To test this hypothesis, we will perform several experiments including measuring the half life of EGFR and determining whether EGFR gets targeted for degradation or if it is recycled after amphiregulin binding. Given that a strong correlation exists between amphiregulin and inflammatory breast cancer, it is important to determine the direct effect of amphiregulin on the genes potentially involved in inflammatory breast cancer development. Amphiregulin has been suggested to activate genes involved in tumor invasion and therefore may play a direct role in the highly invasive and metastatic capacity of inflammatory breast cancer. Therefore, experiments for the third aim will examine the motility and invasive capacity of normal human mammary epithelial cells that have been engineered to express high levels of amphiregulin. In addition, we will determine which genes are turned on in response to amphiregulin binding to EGFR in order to determine how those genes might be important for inflammatory breast cancer progression. This project utilizes the human inflammatory breast cancer cell line SUM149. The use of human inflammatory breast cancer cells should provide data relevant to actual inflammatory breast cancer patient tumors. Our data are expected to define the importance of membrane bound amphiregulin in the activation of EGFR and how an amphiregulin autocrine loop contributes to inflammatory breast cancer progression. This improved knowledge in the progression of breast cancer may ultimately lead to the discovery of a more effective target for therapy.

15. SUBJECT TERMS
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Introduction

Inflammatory breast cancer (IBC) is the most lethal form of primary breast cancer. EGFR is often expressed in IBC and its expression is correlated with a poor prognosis. Amphiregulin (AREG) is one of the seven ligands which binds to and activates EGFR and has recently been shown to be associated with various inflammatory diseases. We developed SUM149 cell line from an aggressive IBC. SUM149 cells have a self-sustaining AREG/EGFR autocrine loop, overexpress AREG mRNA and protein, and are EGF-independent. Recently, we have shown that MCF10A cells growing with AREG are more invasive and motile than MCF10A cells growing with EGF. Additionally, we have shown that AREG results in altered signaling in SUM149 cells including the transcriptional up-regulation of IL-1 α and β and NF-κB activation.

Our studies seek to understand the effect of the AREG in IBC progression. We hypothesize that AREG alters EGFR localization and degradation in IBC. Through confocal microscopy we have shown EGFR accumulation to the cell membrane when AREG is the stimulating ligand, and that Y1045 phosphorylation is decreased after AREG binding.

Body

Accomplishments:

Task 1: To directly test the hypothesis that membrane bound amphiregulin can activate EGFR and is required for maintenance of an amphiregulin autocrine loop

a. Perform juxtacrine growth factor activity assays using SUM149 and MCF10AR cells to determine how membrane bound AREG affects EGFR phosphorylation and proliferation of MCF10A cells grown without EGF.
b. Perform assays using Batimastat as an inhibitor of AREG secretion for an autocrine loop.

Tasks 1a-1b have been completed and published:

Willmarth, N. E. and S. P. Ethier (2006). "Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells." J Biol Chem **281**(49): 37728-37.

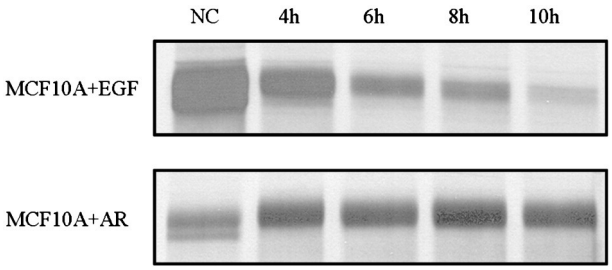
Task 2: To determine how AREG binding to EGFR affects EGFR stability by comparing the recycling and degradation of EGFR in SUM149 cells and MCF10A cells stimulated with AREG or EGF

a. Measurement of EGFR half-life in AREG stimulated cells using a pulse-chase approach
b. Utilize confocal microscopy to determine whether EGFR is localized in either recycling enodsomes or lysosomes after AREG stimulation
c. Determine the affect of AREG on EGFR ubiquitination by measuring phosphorylation of tyrosine 1045, the interaction of c-cbl with EGFR, and ubiquitination of the EGFR after AREG binding

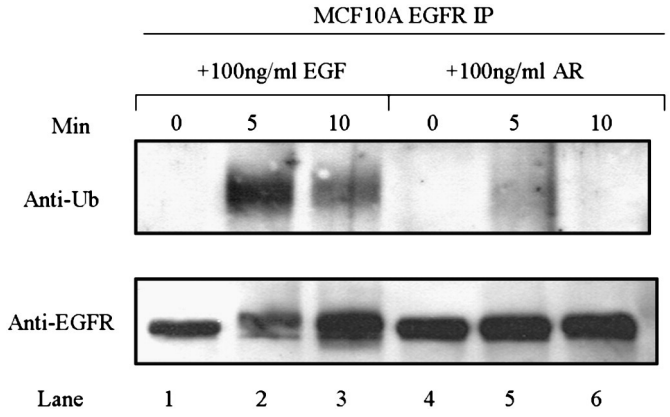
Tasks 2a and 2c have been completed and published:

Willmarth, N. E., A. Baillo, et al. (2009). "Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop." Cell Signal **21**(2): 212-9.

Interaction of AREG with EGFR results in EGFR over-expression and accumulation of EGFR at the cell surface.

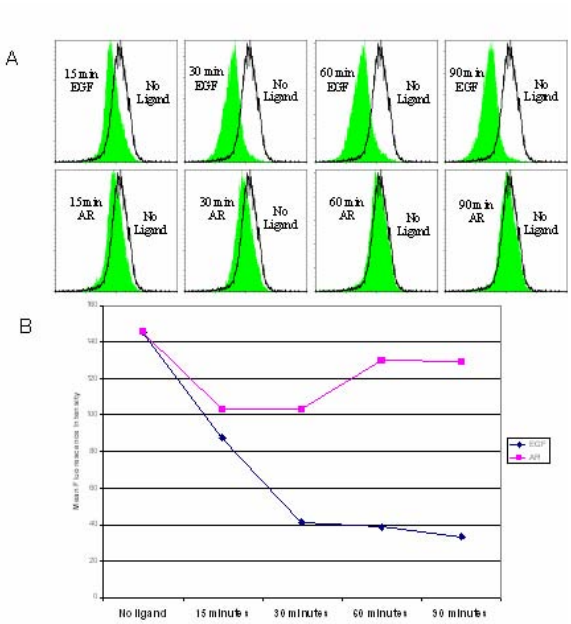


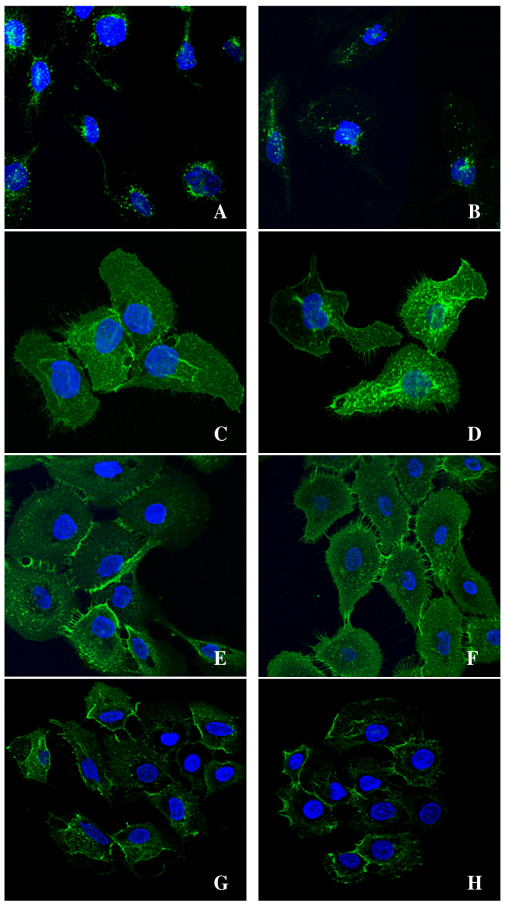
EGFR degradation is decreased in the presence of AR versus EGF during steady-state growth conditions. MCF10A cells were cultured in either 10 ng/ml EGF (1.7 nM) or 20 ng/ml AR (1.8 nM). MCF10A cells were then incubated in methionine/cysteine free media for 1 h. Cells were pulsed with ^{35}S labeled methionine and cysteine for 2 h and then chased with media containing 200× more unlabeled methionine and cysteine for 4, 6, 8, or 10 h. NC are cells that were not chased after ^{35}S incorporation.



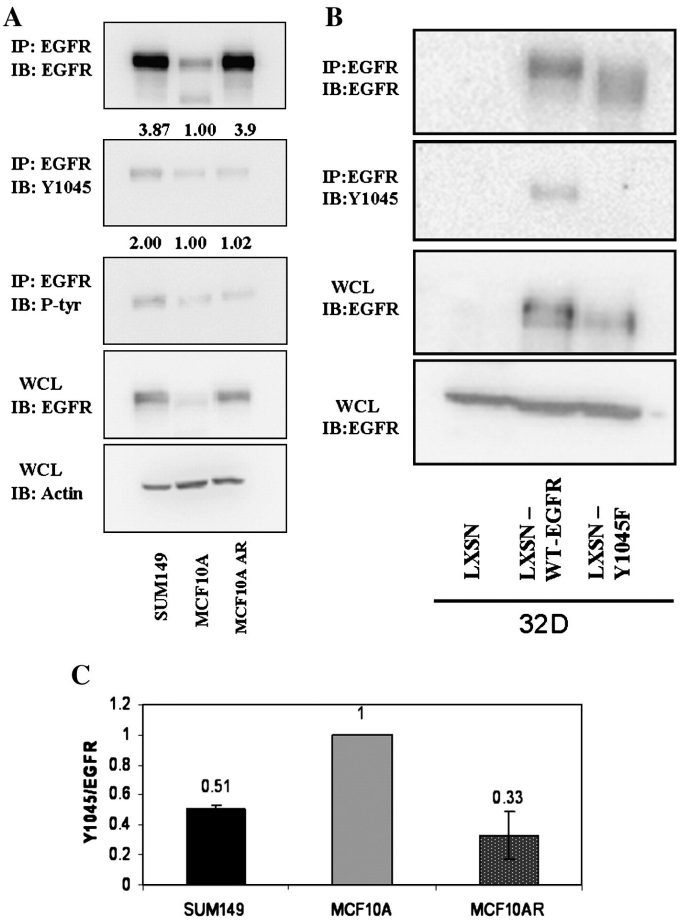
Decreased ubiquitination of EGFR when MCF10A cells are spiked with AR compared with MCF10A cells spiked with EGF. MCF10A cells were deprived of EGF for 24 h and then stimulated with saturating concentrations of EGF and AR (100 ng/ml) for 5 or 10 min. Cells were then lysed and EGFR was immunoprecipitated. Membranes were immunoblotted with either an anti-ubiquitin (anti-Ub) antibody (Cell Signaling) or an anti-EGFR antibody (Zymed).

Impairment of EGFR internalization in the presence of AR. MCF10A cells were deprived of EGF for 24 hours and then spiked with equimolar concentrations of either EGF or AR (16nM) for 15, 30, 60 or 90 minutes. EGFR on the membrane was fluorescently labeled and analyzed using flow cytometry. A) Flow cytometry graphs where the empty peak represents cell surface EGFR before the addition of ligand and the green peaks represent EGFR after ligand is added. B) A graphical representation of the experiments in 3A indicating mean fluorescence intensity over time.





EGFR localization in MCF10A, MCF10A AR, MCF10A + AR, and SUM149 cells. MCF10A (A,B), MCF10A+AR (C,D), MCF10A AR (E,F) and SUM149 (G,H) cells were seeded on coverslips. Cells were then washed, fixed, permeabilized and incubated with anti-EGFR antibody Mab108 for 1 hour followed by incubation with an anti-mouse Alexa 488 conjugated secondary for 1 hour. Coverslips were then mounted on slides and viewed using a 65x water objective on a confocal microscope.



Decreased phosphorylation of EGFR Y1045 when AR is the ligand. A) MCF10A cells, SUM149 cells and MCF10A AR cells were lysed and EGFR was immunoprecipitated using the MAB108 antibody. Membranes were immunoblotted for pY1045 (Cell Signaling), EGFR (Zymed), or p-tyr (Calbiochem). Band densities were quantitated and are displayed relative to MCF10A. Actin was used as a loading control. C) Band density values for pY1045 were divided by the total amount of EGFR relative to MCF10A. This graph displays the average of three different experiments. B) Western blot showing Y1045 phosphorylation of EGFR in 32D myeloid cells transfected with either vector (LXSN), wild type EGFR (LXSN-WT-EGFR) or Y1045 mutant EGFR (LXSN-Y1045F).

Task2b is in progress: To determine EGFR localization EEA1 (to stain the early endosomes) and Rab4 (to stain the recycling endosomes) antibodies will be used with secondary red antibodies to co-stain with EGFR in the cells with and without AREG. Confocal microscopy will then be utilized to look at any co-localization with EGFR to determine the receptors location following AREG stimulation.

Task 3: To determine whether AREG signaling through EGFR contributes to the inflammatory phenotype of IBC.

a. Analysis of motility and invasiveness of MCF10AR cells
b. Expression profiling of MCF10A cells stimulated by either EGF or AREG using microarray technology followed by QPCR and western analysis to confirm microarray results

Tasks 3a-3b have been completed and published:

Willmarth, N. E. and S. P. Ethier (2006). "Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells." J Biol Chem **281**(49): 37728-37.

Reportable Outcomes:

Willmarth, N. E. and S. P. Ethier (2006). "Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells." J Biol Chem **281**(49): 37728-37.

Streicher, K. L., N. E. Willmarth, et al. (2007). "Activation of a nuclear factor kappaB/interleukin-1 positive feedback loop by amphiregulin in human breast cancer cells." Mol Cancer Res **5**(8): 847-61.

Willmarth, N. E., A. Baillo, et al. (2009). "Altered EGFR localization and degradation in human breast cancer cells with an amphiregulin/EGFR autocrine loop." Cell Signal **21**(2): 212-9.

Key Research Accomplishments:

1. SUM149 function through an AREG/EGFR autocrine loop and show an increase in invasiveness and cell motility.
2. AREG alters EGFR internalization and degradation in a way that favors accumulation of EGFR at the cell surface and changes EGFR signaling.
3. IL-1 signaling is required for AREG-induced cell proliferation contributing to IBC progression

Conclusions:

Over the past two years we have begun to understand the role that amphiregulin is playing in IBC. We now understand that membrane bound AREG can activate EGFR and functions through an autocrine loop. Additionally, amphiregulin has been show to increase EGFR stability while decreasing EGFR internalization and degradation. Lastly, we have identified genes, including NF-κB and IL1-α and β that are up regulated in response to AREG which are linked to the increased motility and invasiveness of IBC. From these experiments and future experiments we are learning more about the aggressive phenotypes seen in IBCs.

Future Directions:

In the next year we are going to be knocking down amphiregulin in the SUM149 cells to see if we can reverse the observed aggressive phenotypes. We will continue to look at NF-κB activity and its role in the phenotypes present in the SUM149 cells. We believe that it is the accumulation of EGFR to the cell surface which results in the altered EGFR signaling and NF-κB activation. Mutant forms of EGFR which preferentially recycle to the cell membrane will be used to test these hypotheses.